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MULTIPLE MATING IN THE GLANVILLE FRITILLARY BUTTERFLY: A CASE OF WITHIN-GENERATION BET-HEDGING?

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ABSTRACT

Many hypotheses have been proposed to explain multiple mating in females. One of them is bet-hedging, that is avoiding having no or very few offspring in any given generation, rather than maximizing the expected number of offspring. However, within-generation bet-hedging is generally believed to be an unimportant evolutionary force, except in very small populations. In this study, we derive predictions of the bet-hedging hypothesis for a case in which local insect populations are often small, offspring performance varies e.g. due to inbreeding depression, and the groups of gregarious larvae have to exceed a threshold size before they are likely to survive throughout the larval stage. These conditions exist for populations of the Glanville fritillary butterfly (*Melitaea cinxia*), potentially making bet-hedging benefits larger than usual. We observed matings in a field cage, which allowed detailed observations under practically natural conditions, and analyzed genetic paternity of egg clutches laid by females under direct observation. The egg-laying and survival patterns are in line with the predictions, supporting the hypothesis that multiple mating in *M. cinxia* presents a rare case of within-generation bet-hedging.

KEY WORDS: inbreeding, multiple mating, indirect genetic benefits, compatibility, genetic bet-hedging, *Melitaea cinxia*, metapopulation, microsatellite, last-male precedence pattern

1 Females mate multiply in a wide range of taxa (Birkhead and Møller 1998). Whereas for
2 males reproductive success is expected to increase linearly with the number of mates,
3 the advantages of multiple mating for females are less clear (Jennions and Petrie 2000;
4 Yasui 1997). Mating can be costly to females in terms of time and energy, or because of
5 increased risk of predation, injury or infection (Blanckenhorn et al. 2002; Chapman et al.
6 1995). Multiple mating by females has been explained in terms of direct benefits,
7 particularly in species in which males provide females with a nutrient-rich ejaculate
8 (Arnqvist and Nilsson 2000) or in which resisting mating attempts would be too costly
9 (costs represent the flipside of direct benefits, e.g. (Lee and Hays 2004), and in terms of
10 indirect genetic benefits (Fedorka and Mousseau 2002; Jennions and Petrie 2000;
11 Kozielska et al. 2004; Newcomer et al. 1999).

12 Finally, genetic bet-hedging (Gillespie 1973; Gillespie 1974; Gillespie 1975; Gillespie
13 1977; Hopper 1999; Seger and Brockman 1987) could explain polyandry, especially
14 when females mate indiscriminately (Fox and Rauter 2003; Yasui 1998; Yasui 2001).
15 Multiple mating is a form of bet-hedging because it creates clutches (or groups of
16 clutches) with mixed parentage, as opposed to the monandrous case where a female
17 picks one male to sire all the offspring in all of her clutches. Bet-hedging is often used to
18 describe “adaptive coin-flipping” (Kaplan and Cooper 1984), but in general it is a
19 strategy that reduces the variance in fitness among offspring. It is usually defined in such
20 a way that the mean fitness decreases, and thus variance reduction is the only benefit
21 (Jennions and Petrie 2000; Yasui 1998). In the context of polyandry, the intuitive appeal
22 of bet-hedging is that even if polyandrous females cannot bias paternity patterns, they
23 can hope to avoid the worst-case scenario where all their offspring are fathered by a low-
24 quality or an incompatible (e.g., closely related) male (Fox and Rauter 2003; Jennions
25 and Petrie 2000). This type of bet-hedging serves to avoid the detrimental effects of
26 demographic stochasticity.

27 However, avoiding worst-case scenarios is not a guarantee of evolutionary success.
28 Current theory predicts that bet-hedging is far more likely to be a successful
29 evolutionary strategy when the bets are hedged over several generations, than in a
30 within-generation scenario, to which polyandry belongs (Hopper and Rosenheim 2003;

1 Yasui 1998). In the latter case, the benefits of bet-hedging are predicted to vanish in all
2 but the smallest populations (Hopper and Rosenheim 2003; Yasui 1998).

3 The Glanville fritillary butterfly (*Melitaea cinxia*) occurs in the Åland Islands in SW
4 Finland as a large metapopulation (Hanski 1999; Nieminen et al. 2004). Females mate
5 usually only once, or occasionally twice (Boggs and Nieminen 2004). In 167 and 131
6 wild-caught mated females, only 8% and 6.5% had mated twice based on the count of
7 spermatophores (Kuussaari 1998). In this species, direct benefits of multiple mating are
8 unlikely (see Discussion), but there is a high level of inbreeding and substantial
9 inbreeding depression in small local populations (Haikola et al. 2001; Nieminen et al.
10 2001) Nevertheless, females do not discriminate against close kin as mates (Haikola et
11 al. 2004), possibly because the cost of such discrimination would be too high when there
12 are often very few males locally available (Kokko and Mappes 2005).

13 In order to test whether the bet-hedging hypothesis is likely to explain the occurrence of
14 polyandry in the Glanville fritillary, we first created the theoretical set of conditions and
15 predictions that the system has to show for bet-hedging to apply, and then tested these
16 by determining the reproductive success of singly or multiply mated *M. cinxia*, using
17 material that was collected by intensively observing an experimental population of ca.
18 200 butterflies of equal sex ratio in a large cage in the field (Hanski et al. 2006). This
19 allowed us to quantify multiple mating patterns more precisely than in the field, to detect
20 any post-copulatory paternity-biasing mechanisms, and to tract the number of larvae that
21 survive until diapause.

22 DERIVING THE PREDICTIONS AND CONDITIONS OF THE BET-HEDGING 23 HYPOTHESIS

24 The precise list of predictions made by the bet-hedging hypothesis through polyandry is
25 not readily available from the literature. We shall therefore derive the relevant
26 conditions and predictions here, taking into account the relevant biological features of
27 *M. cinxia*. There are two *a priori* reasons why bet-hedging could be important in this
28 butterfly. Firstly, it persists in the Åland Islands as a metapopulation, in which no single
29 population is safe from extinction within a short period of time (Hanski 1999; Nieminen

1 et al. 2004). Single populations are ephemeral, and they are mostly very small, up to the
 2 point where a single mated female has established an entire local population if she has
 3 mated and lands in an empty patch. This means that evolution favors a reduction in
 4 variance in individual reproductive output.

5 The second reason is related to the overwintering habits of *M. cinxia*. Females lay their
 6 eggs in clusters, and the larvae spin a web on the host plants on which they live. The
 7 larvae diapause as a group, and they tend to remain gregarious until the last molt before
 8 pupating (Kuussaari 1998). An important cause of mortality in natural populations is
 9 overwinter mortality; 20% of the larval groups die during the winter. Overwinter
 10 mortality is known to be dependent on larval group size, and small groups of less than
 11 25 larvae have a very low chance to survive (Kuussaari 1998; Nieminen et al. 2001),
 12 probably because small groups are unable to build a high-quality winter nest, which is
 13 necessary for successful overwintering (Nieminen et al. 2001). Based on data in
 14 Kuussaari (1998), we constructed a logistic regression of the probability of
 15 overwintering survival as a function of group size; the survival probability increases
 16 sharply with the number of pre-diapause larvae (Fig. 1). This result implies that pre-
 17 diapause groups of less than 25 larvae have low overwintering chances; we call this the
 18 Allee threshold size for larval groups.

19 These two observations both improve the prospects of a bet-hedging strategy. Before we
 20 derive the predictions and conditions, let us consider a simple illustrative example where
 21 a hypothetical female butterfly can lay 100 eggs. To do this she may mate
 22 monandrously, polyandrously with two males such that there is no mixed paternity
 23 within one clutch, or polyandrously with two males such that half of offspring in each
 24 clutch are fathered by each male. We consider an illustrative case with two very
 25 different male types. One is largely unsuitable as a mate e.g. due to extreme inbreeding
 26 depression: he gives offspring who survive from summer till diapause with very low
 27 probability $s_1 = 0.05$, the other one yields survival $s_2 = 0.5$. All strategies give the same
 28 mean of offspring production (27.5 in this example), but the variances differ strongly.

29 Under monandry, the expected number of surviving offspring is strongly bimodal, with
 30 variance 521.13. Mixed-paternity polyandry yields a drastic reduction in variance

1 (270.53): in half of the cases she mates with two different males, yielding a peak of
 2 intermediate survival (Fig. 2). The distribution produced by the no-mixed paternity case
 3 depends on the number of clutches. If all eggs are laid in a single clutch, one of the
 4 males yields no paternity at all, and the situation is identical to monandry. However, if
 5 there are two clutches, and the latter may (with 50% probability) be fathered by a
 6 different male, and the outcome is very similar to the case of mixed paternity (Fig. 2).
 7 The variance in this case is the lowest of all three strategies (268.00 in the example of
 8 Fig. 2), and the probability of complete failure (no offspring produced at all) is halved
 9 relative to monandry.

10 All these effects arise without incorporating the Allee threshold. If overwintering
 11 survival chances of offspring increase non-linearly with the number of surviving larvae,
 12 the outcomes will depend strongly on the division of larvae into larval groups: it is
 13 important to have the bulk of the probability distribution of each clutch lie above the
 14 threshold, but if overwintering mortality hits most of larvae in one clutch, it may also be
 15 important to lay several clutches in order to reduce the variance caused by destruction of
 16 whole winter nests, which leads to the demise of all larvae inside (Fig. 1). Since this
 17 trade-off between individual clutch sizes and the total number of clutches is difficult to
 18 visualize in Fig. 2, we will now leave this simplified example and turn our attention to a
 19 more systematic investigation of possible fitness consequences with different mating and
 20 clutch size strategies.

21 Fig. 3 examines predictions for five different fitness components for females, who mate
 22 singly, multiply but with only one sire per clutch, and multiply with mixed paternity
 23 (two males with 50% each) in each clutch. Fitness components are derived assuming
 24 that there are two types of males, and the pre-diapause survival of each egg (i.e. from
 25 egg-laying until the overwintering nest is built) is s_1 if the sire is of type 1 and s_2 if the
 26 sire is of type 2. As before, these could reflect e.g. related and unrelated males, the
 27 former yielding low survival of offspring due to inbreeding depression (Haikola et al.
 28 2004; Keller and Waller 2002). The pre-diapause survival of each egg is assumed to be
 29 independent of the survival of other eggs, thus the number of pre-diapause larvae is
 30 binomially distributed. This assumption is not entirely true, as there is in *M. cinxia* a

1 consistently positive effect of group size on survival throughout development from egg
 2 stage to the last caterpillar instar (Kuussaari et al. 2004). However, due to scarcity of
 3 data we have not quantified this relationship, and assuming density independence will
 4 give us conservative estimates of the prospects of bet-hedging (as including it would add
 5 another Allee effect). Survival through diapause is then assumed to depend on the
 6 presence of other larvae as in Fig. 1. In the examples of Fig. 3, a female is assumed to
 7 have a total budget of 400 eggs, and she can lay them in 1 to 6 equal clutches (i.e., in
 8 batches of 400, 200, 133, 100, 80 or 67 eggs, respectively).

9 Regardless of the mating strategy, the simple arithmetic mean of post-diapause offspring
 10 number decreases with an increasing number of clutches (Fig. 3a), together with the
 11 decline in the survival of individual clutches (Fig. 3b) due to the smaller size of each
 12 individual clutch. However, the expected number of surviving clutches increases when
 13 there are many clutches (Fig. 3c). Such ‘safety in numbers’ in terms of numbers of
 14 clutches shows a trade-off with ‘safety in numbers’ operating within a clutch. The
 15 probability of complete failure, i.e. no surviving post-diapause offspring, increases with
 16 the number of clutches which the eggs are divided into (Fig. 3d), reflecting the intense
 17 within-group Allee effect of overwintering survival in small groups. Finally, multiple
 18 mating reduces the variance in post-diapause offspring numbers significantly (Fig. 3e),
 19 but to achieve this effect when paternity is not mixed, the female should also decrease
 20 her clutch size.

21 In a species with population dynamics as complicated as in *M. cinxia* (Hanski et al.
 22 1995) it is not easy to determine which fitness component best reflects success in nature:
 23 maximizing those in Fig. 3a-c, minimizing complete failure, Fig. 3d, or minimizing
 24 variance, Fig. 3e. In reality, the best strategy is likely to balance some of the benefits of
 25 each measure. Keeping this in mind, we can obtain several qualitatively robust results
 26 from Fig. 3. In the following we list these as two ‘predictions’ and three ‘conditions’ that
 27 must be met for bet-hedging to apply.

28 If bet-hedging is unimportant and individuals simply maximize the expected number of
 29 post-diapause offspring, selection always favours laying eggs in as few and large
 30 clutches as possible (Fig. 3a), where ‘as possible’ should be considered taking into

1 account physiological limits and local availability of larval food sources. However, if
 2 clutches also experience random mortality (irrespective of their size) that kills all
 3 offspring in one clutch, it should become beneficial to bet-hedge by enlarging the
 4 expected number of clutches that survive until the next generation. Bet-hedging thus
 5 predicts that clutch sizes will reflect a trade-off between the decrease in Fig. 3a and the
 6 increase in Fig. 3c, i.e. some intermediate choice. However, this type of bet-hedging
 7 relies on random (independent) mortality across clutches, and does not predict that the
 8 clutch sizes of monandrous and polyandrous females would differ: the fitness
 9 components do not differ much between the mating strategies. We thus arrive at our first
 10 prediction:

11 *Prediction 1.* If females do not bet-hedge at all, they should lay as large clutches as
 12 possible given the constraints that operate on e.g. female physiology and larval ecology.
 13 But if they bet-hedge because of random mortality hitting individual clutches, they
 14 should lay more and smaller clutches. This prediction applies across all mating
 15 strategies.

16 Prediction 1 may be hard to test because it is difficult to know about all potential
 17 constraints, and because it does not predict strong differences between female mating
 18 strategies. In contrast, the mating strategy has a large effect on the probability of total
 19 failure (Fig. 3d) and on the variance in the number of post-diapause offspring (Fig. 3e).
 20 Monandrous females experience a much greater probability of failure, in particular if
 21 they lay many small clutches. All their clutches are necessarily genetically similar, and if
 22 they have mated with a male who yields few surviving offspring, then all clutches are
 23 likely to fail due to the within-group Allee effect. They can thus only avoid a significant
 24 risk of complete failure (Fig. 3d) if they lay many eggs in a single clutch (in the hope
 25 that enough survive above the Allee threshold). For multiply mating females that mix
 26 paternity within broods, the risk of total failure is smaller (Fig. 3d) and they can achieve
 27 low variance in the number of post-diapause offspring even if they do not lay many
 28 clutches (Fig. 3e). If mixing paternity is not an option, however, females need to gain the
 29 bet-hedging benefit by laying many clutches: some will then be sired by very suitable

1 males, others by very unsuitable ones, and the total risk of going below the Allee
2 threshold in every clutch remains small.

3 It follows that if the fitness components of minimizing total failure or the variance are
4 important, we predict them to have a much stronger selection for monandrous females to
5 increase their clutch sizes than for polyandrous ones, and polyandrous females are
6 instead selected to increase the number of clutches particularly if they cannot mix sperm
7 within clutches. The risk reduction through multiple mating frees these females to reap
8 the advantages of other fitness components, such as that outlined in prediction 1. We
9 arrive at our second prediction:

10 *Prediction 2.* If bet-hedging to avoid the within-group Allee effect is an important
11 component of fitness, we predict differences between mating strategies in the size and
12 number of clutches. Monandrous females should lay few large clutches, while
13 polyandrous females gain the bet-hedging benefit by laying several small clutches. The
14 difference between polyandrous females that mix paternity within clutches and those
15 that do not is predicted to be small, but in case of a difference, females that produce
16 clutches of non-mixed paternity should have the smallest clutches.

17 Our Fig. 3 illustrates these predictions using single numerical examples only, but other
18 numerical choices lead to qualitatively identical conclusions: in particular, we created
19 100 random values for s_1 and s_2 between 0.05 and 0.95, and checked the number of
20 clutches that minimizes the variance for each mating strategy. Monandrous females were
21 always favoured to lay a smaller (64% of cases) or identical (36%) number of clutches
22 than females with non-mixed paternity polyandry, never a larger one. Mixed-paternity
23 polyandry had more variable optima, that were below the optimum of monandrous
24 females in 18% of cases, identical to them in 74% of cases, and larger in 8%. Thus the
25 mixed-paternity strategy yields a clearer prediction regarding variance reduction in
26 general than a directional selection on clutch size (Fig. 3e), while our prediction that
27 differences in clutch size strategy reflect differences in Fig. 3d-e remains very robust if
28 females usually cannot mix paternity within broods.

1 All fitness components become identical across all mating strategies if $s_1 = s_2$. This
 2 result relates to the first of three additional conditions that the mating system has to
 3 fulfill for the bet-hedging hypothesis to apply:

4 *Condition 1.* Explaining multiple mating through bet-hedging requires showing that
 5 males vary in their ability to produce viable offspring with a given female.

6 Note that this does not require that males can be ranked in an order of genetic quality;
 7 compatibility issues such as inbreeding, that vary from female to female, are sufficient.

8 There are two additional, general conditions of the bet-hedging hypothesis:

9 *Condition 2.* Polyandrous females should have a lower variance in the number of
 10 surviving offspring.

11 This is a direct reflection of the nature of the bet-hedging hypothesis in general (Yasui
 12 1998).

13 *Condition 3.* All other factors being equal, polyandrous females should run a lower risk
 14 of all their clutches falling below the Allee threshold.

15 This last condition may be difficult to test, since all other factors are not expected to be
 16 equal. If monandrous females optimize their reproduction and lay fewer clutches, the net
 17 risk for both types of females can become rather similar (e.g. compare 3 clutches for
 18 monandrous females with 6 for polyandrous, Fig. 3d). However, if multiple mating
 19 evolves for the reason that it reduces the risk of total failure, it should logically retain a
 20 net benefit.

21

22 MATERIAL AND METHODS

23 *Field cage experiment*

24 Post-diapause larvae were collected in the spring 2003 from 40 local populations in the
 25 Åland Islands in SW Finland and reared under common garden conditions in the

1 laboratory. Pupal weight was used as a measure of body size. Altogether 194 newly-
 2 eclosed butterflies (81 females and 113 males) were sexed, marked and released into a
 3 32 by 26 by 3 m field cage covered with mesh. The cage had been constructed on a
 4 natural dry meadow. The cage and the experiment conducted in it are described in
 5 (Hanski et al. 2006). Natural flowers occurring at the site provided nectar to butterflies,
 6 while 250 larval host plants (*Plantago lanceolata*) in flower pots were placed in the cage
 7 for ovipositing females. Prior to their release, a small piece was removed from the hind
 8 wings of each butterfly for DNA analysis. Matings and ovipositions were carefully
 9 recorded, but a small fraction was nonetheless missed, which became evident as the host
 10 plants were checked every evening for egg-clutches. The egg clutches were removed
 11 immediately after oviposition, and they were reared in the laboratory until diapause.
 12 Larvae were counted soon after hatching to determine egg hatching rate, and they were
 13 counted again just before diapause to determine pre-diapause larval survival. The fact
 14 that the eggs were brought into the laboratory for hatching and rearing removes all
 15 environmental effects except for maternal-paternal pairings, a necessary condition for
 16 evaluating whether males vary in their ability to sire young.

17 Out of the 67 females that were recorded to lay fertile eggs, 18 had not been observed to
 18 mate (Hanski et al. 2006). Most of the ovipositions were detected, as only 32 additional
 19 egg clutches were discovered by inspecting the host plants in the evening, while 225
 20 ovipositions were recorded directly. Multiple mating was observed relatively frequently:
 21 14 females were recorded mating twice, and 4 females mated three times. 31 females
 22 were observed to mate once.

23 *Parentage analysis*

24 All the 194 butterflies that were released into the cage were genotyped. Of the 18
 25 females that had been recorded to mate multiply, 11 females produced enough offspring
 26 after the second mating to be used for parentage analysis. Altogether 354 larvae from 32
 27 clutches laid by these 11 females were analyzed. For each clutch, 5 to 12 larvae were
 28 genotyped. Genomic DNA was extracted and amplified with polymerase chain reaction
 29 (PCR) using the primers described by (Sarhan 2006). Details of the PCR protocols and
 30 the cloning and characterisation of the microsatellite loci are given in (Sarhan 2006).

1 The PCR products were run on an ABI Prism 377 automated sequencer (Perkin-Elmer)
 2 and the alleles were scored using the Genotyper software (Applied Biosystems).
 3 Amplification products from five primers allowed unambiguous paternity assignments.

4 *Data analysis*

5 The data were analyzed using generalized linear models using the R Software
 6 (RDevelopmentCoreTeam 2005). The significance of a variable was tested based on the
 7 difference in deviance between a model with and without that variable, using *F*-tests. All
 8 females that had not been observed mating but produced eggs that hatched successfully
 9 were considered monandrous (unmated females can produce only sterile eggs). They
 10 were obviously not included while evaluating the significance of male identity. For some
 11 of the egg clutches, the number of hatched larvae had not been recorded, and these
 12 clutches were removed from the analysis of late-larval survival. Out of the 18
 13 polyandrous females, 2 females had to be removed from all analyses because of missing
 14 records of the number of diapause larvae.

15 When analyzing the influence of female mating status (monandrous versus polyandrous)
 16 on clutch size, first clutch size (when laid before remating), and best clutch size, we
 17 assumed a quasi-poisson distribution. The influence of female mating type on the size of
 18 the largest clutch produced was evaluated by comparing a model with that variable to a
 19 model where the life-time egg-production was the only explanatory variable. In the
 20 analyses of clutch number, life-time egg production, number of diapause larvae per
 21 clutch and total number of diapause larvae, all egg clutches produced by the same
 22 female were pooled, and we assumed a quasi-poisson distribution. For the analysis of
 23 egg hatching rate, late-larval survival (survival from hatched larvae until diapause), total
 24 larval survival (from egg to diapause larvae), female remating propensity, and likelihood
 25 for a male to be chosen by a mated female, we assumed a quasibinomial distribution.
 26 Because females produce several egg clutches during their lifetime that tend to become
 27 smaller with time, female identity was used as random factor and clutch rank was used
 28 as an independent variable in all analyses where egg clutches were not pooled, and the
 29 significance of female mating type was evaluated based on the difference between a
 30 model with and without that variable. Similarly, the influence of male identity on the

1 same dependent variables was evaluated by comparing a model including this variable to
2 a model with only female identity and clutch rank as independent variables.

3 Finally, the total number of diapause larvae was regressed against life-time egg
4 production separately for monandrous and polyandrous females, and an F -test to
5 compare two variances was used to test for a difference in variance in the residuals
6 between monandrous and polyandrous females.

7 To test whether males are sperm limited, we analyzed whether the number of times a
8 male had mated previously and the time since its last mating had an influence on the
9 total number of eggs the female will produce, the egg hatching rate and larval survival,
10 and whether the female will remate. To assess the trading up hypothesis, we analyzed
11 whether males that mated females chose to remate with were different from other males
12 in the population (age, weight, total number of matings achieved, total number of
13 offspring produced). We also tested, using logistic regression, whether the probability
14 that the clutch exceeds the Allee threshold at the pre-diapause stage depends on the
15 female's mating status.

16 RESULTS

17 *Parentage Analysis*

18 Out of the 32 polyandrous clutches analyzed, 4 were produced before the second mating,
19 and hence the first male was the only possible father. This was confirmed by the genetic
20 analysis. In 18 clutches out of the 28 remaining, the last male to mate with the female
21 fathered all of the offspring. This includes all the 3 clutches that were produced by
22 females after third matings. For one egg-clutch, the genetic analysis revealed that it had
23 mistakenly been attributed to a particular female. This egg-clutch was removed from all
24 further analyses. There were 9 egg-clutches produced by five females that were not
25 entirely sired by the last male they mated with. These exceptions to the last-male sperm
26 precedence could not be explained by the age of the males, number and timing of
27 previous matings or time span between matings.

28

Multiple matings: testing the predictions

The total number of eggs produced did not differ significantly between monandrous and polyandrous females (404.16 ± 237.74 and 401 ± 215.66 , respectively, $F_{1,63} = 0.002$, $p = 0.963$). Egg-hatching rate and offspring survival were not affected by the female's number of matings: mean offspring survival from egg to diapause was similar for both groups ($F_{1,66} = 1.53$, $p = 0.22$). There was no difference between monandrous and polyandrous females in the total number of offspring surviving until diapause (131.35 ± 101.90 and 147.5 ± 91.72 , respectively, $F_{1,63} = 0.309$, $p = 0.580$).

Polyandrous females produced smaller clutches than monandrous females (90.14 ± 62.92 and 135.64 ± 63.15 , respectively, $F_{1,66} = 6.34$, $p = 0.014$) and they divided their fecundity into significantly more clutches than monandrous females (4.38 ± 2.99 and 2.98 ± 1.57 , respectively, $F_{1,63} = 5.94$, $p = 0.018$). Females that produced clutches with mixed paternity were not significantly different in this respect from polyandrous females that were not recorded to have mixed clutches, although the sample size is small with only 3 females with mixed paternity (altogether 6 clutches). In 12 out of 16 cases the female who eventually became polyandrous had not yet mated more than once when producing the first clutch, and they laid smaller first clutches than monandrous females (122.9 ± 67.59 and 169.4 ± 63.19 , $F_{1,59} = 5.007$, $p = 0.029$).

Clutches of females whose matings were observed could be classified in five categories: (A) laid by a singly mated female ($n = 87$); (B) laid by a multiply mated female, 100% of eggs fertilized by the 1st male ($n = 20$); (C) laid by a multiply mated female, 100% of eggs fertilized by a later male than the 1st ($n = 15$); (D) clutch laid by a multiply mated female, containing mixed paternity ($n = 6$); and (E) laid by a multiply mated female, and paternity distribution unknown ($n = 33$). Pooling all clutches within each category, the probability of exceeding the Allee threshold (at least 25 offspring surviving until pre-diapause stage) increased significantly with clutch size in each category (Fig. 4). Including female identity as a random factor (generalized linear mixed model with binomial error), we could not detect significant differences between the logistic regressions, apart from a non-significant tendency ($p = 0.06$) that clutches with 100% paternity by a later male exceed the Allee threshold more easily than those sired by the

1 first male of a polyandrous female (statistics given in Fig. 4). Applying a Bonferroni
2 correction would move this value of p further away from significance.

3 To test the prediction that the variance in the number of surviving offspring is smaller
4 for polyandrous females, we needed to correct for the fact that variance increases with
5 the number of eggs laid. The variance of the residuals in number of offspring surviving
6 until diapause (regressed against eggs laid) was higher for females that had mated only
7 once relative to females that had mated several times (Fig. 5a, F test to compare two
8 variances: $F_{15,48} = 0.37$, $p = 0.04$). The number of offspring surviving in the female's
9 best clutch was higher in females that had mated several times than in females that had
10 mated only once, for any given clutch size (Fig. 5b, $F_{1,63} = 6.806$, $p = 0.011$). Out of 16
11 multiply mated females, none failed to produce at least one pre-diapause clutch that
12 exceeded the Allee threshold. Of the monandrous females, this number was six out of 66
13 (9.1%). This is a non-significant trend in the predicted direction (Fisher's exact test, one-
14 tailed $P = 0.26$).

15 Male identity had an influence on the number of hatched larvae ($F_{12,97} = 3.63$, $p =$
16 0.0002), and the number of diapause larvae ($F_{12,107} = 2.91$, $p = 0.002$), and it also
17 influenced the egg hatching rate ($F_{12,97} = 3.56$, $p = 0.0003$). Although male identity had
18 no influence on late larval survival (i.e. survival from hatched larvae to diapause larvae)
19 $F_{10,82} = 0.51$, $p = 0.878$), it had a significant influence on the total larval survival of the
20 clutch (from egg to diapause larvae) ($F_{12,107} = 2.46$, $p = 0.008$).

21 The propensity of females to remate was not affected by the male's previous number of
22 matings ($F_{1,62} = 0.30$, $p = 0.588$), the time since the male's last mating ($F_{1,18} = 0.34$, $p =$
23 0.568) or the male's age ($F_{1,62} = 0.411$, $p = 0.52$). Males that mated females chose to
24 remate with were not different from other males with respect to weight ($F_{1,52} = 1.16$, $p =$
25 0.287), total number of matings achieved ($F_{1,52} = 0.01$, $p = 0.911$), total number of
26 offspring produced ($F_{1,52} = 0.07$, $p = 0.796$), offspring egg hatching rate ($F_{1,52} = 1.10$, $p =$
27 0.299) or offspring survival ($F_{1,52} = 1.40$, $p = 0.242$). However, they were on average
28 younger than the other males present in the cage at the time of the mating ($F_{1,79} = 6.27$, p
29 $= 0.014$).

1 The number of eggs a female laid was not affected by the male's age ($F_{1,78} = 0.61$, $p =$
 2 0.44) or the time elapsed since its last mating ($F_{1,25} = 1.57$, $p = 0.22$). Egg hatching rate
 3 and larval survival were not affected by the male's age (egg hatching rate: $F_{1,56} = 0.08$, $p =$
 4 0.78, larval survival: $F_{1,51} = 1.93$, $p = 0.28$), previous number of matings (egg hatching
 5 rate: $F_{1,56} = 0.32$, $p = 0.57$, larval survival: $F_{1,51} = 1.82$, $p = 0.18$) or time elapsed since
 6 last mating (egg hatching rate: $F_{1,20} = 1.13$, $p = 0.30$, larval survival: $F_{1,19} = 0.43$, $p =$
 7 0.52).

8 DISCUSSION

9 Within-generation bet-hedging, that is reducing the variance in the number of offspring
 10 to avoid having no or very few offspring in any given generation, is theoretically
 11 difficult to maintain if it comes at a cost of not maximizing the expected number of
 12 offspring. We found that clutches of polyandrous females had a significantly lower
 13 variance than monandrous females in the number of offspring that survive to reach
 14 diapause. Thus, polyandrous females appeared to benefit of bet-hedging. However, bet-
 15 hedging did not appear to come at a cost of reduced total number of offspring surviving
 16 to diapause. Below, we discuss why *M. cinxia* can be particularly prone to experience
 17 advantages of within-generation bet-hedging, without having to pay the associated cost.

18 The first reason why bet-hedging can be important is the metapopulation structure of *M.*
 19 *cinxia* in the Åland Islands (Hanski 1999; Nieminen et al. 2004). Local populations are
 20 small, often extremely so: females disperse only after they have mated, and may singly
 21 found an entire new local population (Hanski 1999; Hanski et al. 1995). Ensuring that at
 22 least some offspring are viable through variance reduction is then a valid argument in
 23 favour of bet-hedging (Yasui 1998). This can be achieved either by mating multiply
 24 before dispersal, or alternatively in the new patch if new males are encountered there. In
 25 this latter case the opportunity to reduce the variance arises after dispersal, but the same
 26 logic still applies, as long as the local populations remain small.

27 How strong is our evidence for bet-hedging in *M. cinxia*? Of the two predictions and
 28 three conditions we derived for within-generation bet-hedging, our data are in full
 29 agreement with both predictions and two of the conditions. Condition 3, that females

1 should run a lower risk of all their clutches falling below the Allee threshold, was only
2 supported in the sense of a non-significant trend.

3 The egg-laying pattern is particularly intriguing. Polyandrous females laid smaller
4 clutches, and importantly, they did so already before they had mated twice — which is
5 consistent with the idea that polyandry is a form of bet-hedging strategy that manifests
6 itself already before the female has found another mate, rather than a chance event that
7 occurs for some females, possibly controlled by male rather than female behaviour.
8 Consequently, we consider the alternative that the mating pattern is a result of male
9 manipulation (Arnqvist and Rowe 2005) unlikely, and we definitely detected no cost of
10 multiple mating in terms of reduced lifetime reproductive success or other measures of
11 fitness. Another potential form of sexual conflict occurs when males prevent remating:
12 in butterflies, mating plugs are widespread (Ehrlich and Ehrlich 1978) and male
13 ejaculate often contains apyrene sperm that is known to influence female receptivity by
14 filling the spermatheca and delaying female remating (Cook and Wedell 1999). *Melitaea*
15 *cinxia* shows no obvious evidence of a mating plug (Wahlberg 1995), but we cannot
16 exclude the possibility that males could influence female remating in more subtle ways.
17 However, support for such a hypothesis was not found either, as male identity had no
18 clear influence on the future mating behaviour of the female.

19 Since bet-hedging individuals do not strive to maximize arithmetic mean fitness, they
20 are often expected to pay a cost in terms of this fitness measure (Hopper and Rosenheim
21 2003): indeed, theoretical results of bet-hedging often phrase it as a trade-off between
22 mean and variance of offspring numbers (Gillespie 1974; Gillespie 1975; Gillespie
23 1977; Proulx 2000). Therefore, all other factors being equal, multiply mating females
24 should have a lower total number of surviving offspring (Fig. 3a). Our failure to find this
25 cost indicate that there are additional factors that play a role in determining the fitness of
26 bet-hedging females. There is nothing in the definition of bet-hedging that prohibits it
27 from co-occurring with other benefits of multiple mating. Among possible benefits that
28 we cannot completely exclude is that polyandrous females gained additional fitness
29 through a ‘trade-up’ mechanism. Females can be particularly likely to compensate by
30 remating with genetically superior (Hasselquist et al. 1996; Kempnaers et al. 1997;

1 Pitcher et al. 2003) or more compatible males (Garner and Schmidt 2003; Masters et al.
 2 2003) if their first mate is somehow found unsatisfactory. This could in principle explain
 3 the similar total number of surviving offspring between monandrous and polyandrous
 4 females (including a trend of more offspring for polyandrous females). Indeed, later
 5 sires showed a marginally significant tendency to produce better results, in terms of
 6 exceeding the Allee threshold, than first sires, although lack of power due to low sample
 7 size of confirmed paternity in later clutches warrants caution in applying this argument.

8 Direct benefits appear less likely as an alternative explanation, as polyandrous females
 9 did not lay more eggs. While there is evidence that multiple mating in insects is often
 10 related to direct benefits, the effects found are typically very small (Fox 1993; Torres-
 11 Vila et al. 2004). In the closely related *Euphydryas editha*, variation in spermatophore
 12 size had no significant effect on female reproductive output (Jones et al. 1986) and
 13 spermatophores are unlikely to function as nuptial gifts in *M. cinxia* either (Boggs and
 14 Nieminen 2004). Females could also remate to ensure a sperm supply. Even though
 15 sperm production can be limited in males (Olsson et al. 1997), *M. cinxia* males do not
 16 seem to be sperm limited, even after several matings or when the time between matings
 17 is very short.

18 Regarding condition 3 which, as must be stressed, was not supported with full statistical
 19 significance, it is also worth pointing out a feature of the experimental setup that causes
 20 it to underestimate the variance in male compatibility and hence the importance of bet-
 21 hedging. The butterflies originated from large, well-connected populations, and were
 22 thus not expected to be inbred. In natural conditions, there is high inbreeding depression
 23 in small populations, and high relatedness between the parents causes an important and
 24 significant decrease in offspring egg-hatching rate and survival (A. Sarhan and S.
 25 Haikola, in prep.). Previous results show that relatedness between the parents is much
 26 more important in determining offspring fitness than either parent's heterozygosity (A.
 27 Sarhan and S. Haikola, in prep.). If the indiscriminate female mating behaviour extends
 28 to accepting related males, as shown in Haikola et al. (2004), and often expected to be
 29 adaptive when males are encountered sequentially (Kokko and Ots 2006), the variance

1 in offspring fitness would be much higher for monandrous females, and the difference
2 between monandrous and polyandrous females would be similarly magnified.

3 Finally, a particular feature in the biology of *M. cinxia* means that the prospects for
4 finding a significant evolutionary advantage of bet-hedging could be much elevated for
5 this species in the particular case of mixed paternity in a single clutch of eggs.

6 Overwintering in communal nests means that it is very important for a female to have at
7 least one clutch that has at least 25 larvae at pre-diapause. The non-independence of
8 larval survival means that mixing paternity can be advantageous for guaranteeing that at
9 least some offspring in each clutch are highly viable, and the clutch as a whole therefore
10 exceeds the required threshold. Unfortunately, this specific benefit of bet-hedging was
11 difficult to test in our dataset that yielded few clutches of mixed paternity. These appear
12 to perform very well in exceeding the Allee threshold even at small clutch sizes
13 (category D in Fig. 4), but statistical significance is lacking. How likely this factor is to
14 influence bet-hedging strategies in nature will depend on how often females have control
15 over paternity, above the usual pattern of last male sperm precedence (Bonduriansky
16 2001; Eady et al. 2004).

17 Finally, it must be kept in mind that there are multiple explanations of multiple mating,
18 which makes it impossible to falsify all alternatives with a single study. Nevertheless,
19 both the intriguing egg-laying pattern of polyandrous females and the resulting lowered
20 variance in their life-time reproductive success suggest that within-generation bet-
21 hedging is in this case an unusually likely candidate for explaining the occurrence of
22 polyandry as a successful evolutionary strategy. This obviously begs the question why
23 polyandry has not spread to fixation: theory shows that in a metapopulation with local
24 density regulation selection against demographic stochasticity can be strong even though
25 the entire population is large (Proulx 2000). There are several potential explanations,
26 one of which is the need to produce many clutches which necessitates a sufficiently long
27 lifespan. Whether such a cost is significant in nature, remains to be tested. Our cage
28 setup did not detect such a cost at the level of lifetime fecundity, even though predation
29 was possible in the cage. Birds were obviously excluded from the cage setup, but
30 checkerspot sequestrate chemical compounds and they rarely suffer predation from birds

1 (Nieminen et al. 2004). Predation by spiders and predatory insects is a much more
 2 important cause of mortality in *M. cinxia* (Nieminen et al. 2004), and spiders were very
 3 abundant in the cage (M. Saastamoinen, personal communication).

4 Alternative possibilities include the metapopulation structure of *M. cinxia* that consists
 5 of patches that differ in connectivity. This has been found to create spatial variation in
 6 other traits such as dispersal and the associated fecundity (Haag et al. 2005; Hanski et al.
 7 2006), and mating strategies could also conceivably differ between well connected
 8 populations and small, isolated ones. The conditions of bet-hedging are generally better
 9 met in the latter, which creates interesting possibilities of spatially varying selection.

10

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- 23

FIGURE LEGENDS

Fig. 1. Logistic regression of the probability that a clutch survives the winter, from data presented in (Kuussaari 1998). The regression is given by (probability of survival) = $[1 + \exp(1.9219 - 0.079n)]^{-1}$, when the pre-diapause nest contains n larvae ($\beta = 0.079$, $\chi^2 = 19.83$, $df = 1$, $P < 0.001$). Below the Allee threshold of 25 larvae, successful overwintering is highly unlikely.

Fig. 2. A hypothetical example where a female has 100 eggs, and there are two equally common male types, giving offspring with survival $s_1 = 0.05$ or $s_2 = 0.5$. The survival of each offspring until diapause is assumed to be independent of the survival of others, and neither monandrous nor polyandrous females can detect male type. Thus polyandrous females are assumed to mate twice but in 50% of cases this means remating with the same male type. Polyandrous females can opt to lay two clutches 50 eggs each (one for each mate) instead of one clutch, though the result only differs in the case of no-mixed paternity polyandry. See text for details on the variances in the number of surviving offspring.

Fig. 3. Predicted values of various fitness components for different mating strategies. We assume two potential sires, one giving survival from egg to diapause s_1 for each egg, the other s_2 . Fitness components are then calculated assuming monandry (open circles), polyandry with no mixed paternity (crosses), and polyandry with each male siring 50% of offspring (stars), and the total egg budget is 400 eggs for each female, divided into 1 to 6 clutches. The mean number of post-diapause offspring (a) is obtained by first computing the weighted sum of binomial distributions of the number of pre-diapause offspring (weights indicate the probability of mating with either type of male, e.g., a monandrous butterfly has 50% of chance of survival s_1 for each egg and 50% of s_2), and then assuming that each clutch survives the winter independently from other clutches with a probability that depends on clutch size (b), as indicated in the logistic regression of Fig. 1. Both the expected number of surviving post-diapause clutches (c) and the probability of complete failure (d, the probability that there are no clutches that survive the winter) increase with the number of clutches. Variance (e, indicated as standard

1 deviation of the number of post-diapause larvae) is reduced by bet-hedging, but in the
 2 case of no mixing of paternity, this requires increasing the clutch size. The examples are
 3 derived using $s_1 = 0.25$, $s_2 = 0.45$.

4 Fig. 4. Logistic regressions for the probability of exceeding the Allee threshold at pre-
 5 diapause for clutches of type A (circles), B (small filled squares), C (stars), D (open
 6 triangles), and E (filled dots); see main text for definitions of clutch type. The increase
 7 of probability with clutch size is highly significant ($p < 0.001$) for all types except
 8 clutches of mixed paternity (type D, $p = 0.16$, $n = 6$). However, the differences between
 9 the regressions are not significant in a mixed model (logistic regression) with female
 10 identity as a random factor. In particular, there is no significant difference between
 11 categories B (“first male of polyandrous females”) and C, D, or E (“later male”), either
 12 when C, D, and E were pooled to constitute one category, or when comparing separately
 13 with B: significance of the category was $p > 0.2$ in each case except for the comparison
 14 between B-C, in which case there was too little data to keep female identity as a random
 15 factor, and ignoring it produced a non-significant tendency ($p = 0.06$) that small clutches
 16 100% sired by a later male exceed the Allee threshold more easily than equally small
 17 clutches sired by the 1st male.

18 Fig.5. Offspring fitness (n) for monandrous and polyandrous females. A. Number of
 19 diapause larvae regressed against total number of eggs produced by each female. B.
 20 Fitness of each female’s best clutch regressed against clutch size. Open circles:
 21 monandrous females; solid circles: polyandrous females.

22

23

24

FIGURES

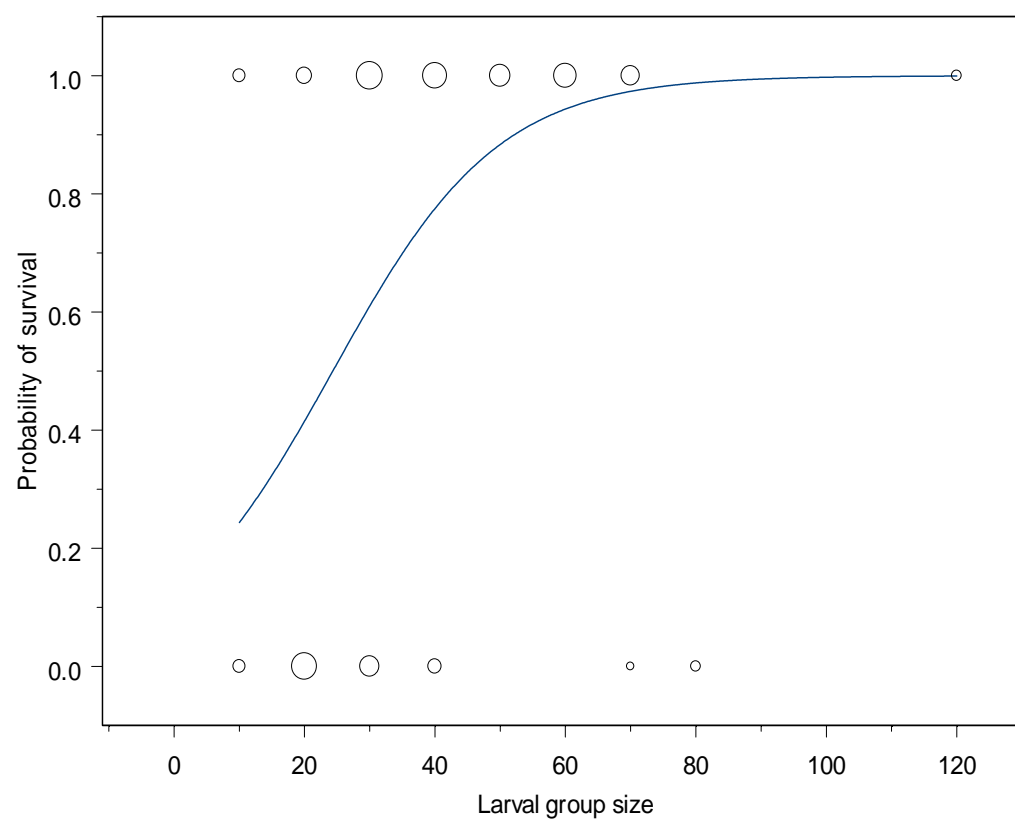


Fig. 1

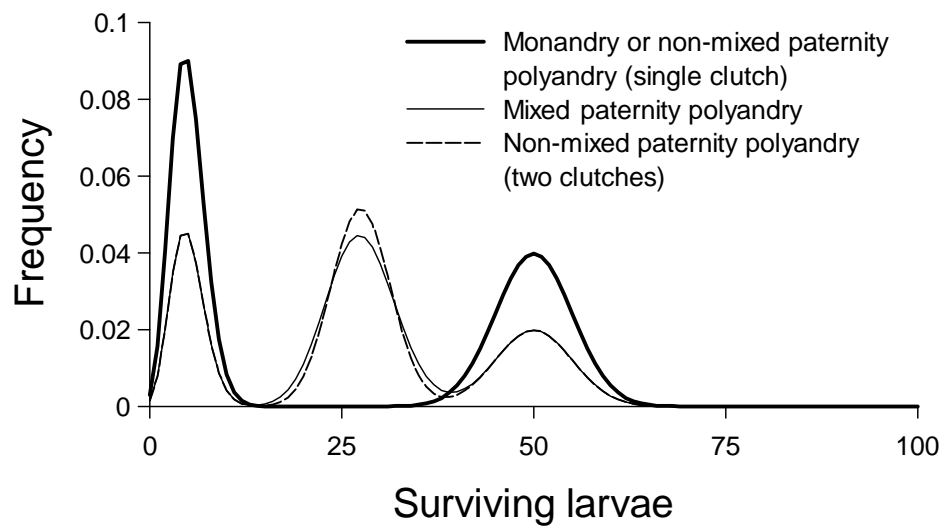


Fig. 2.

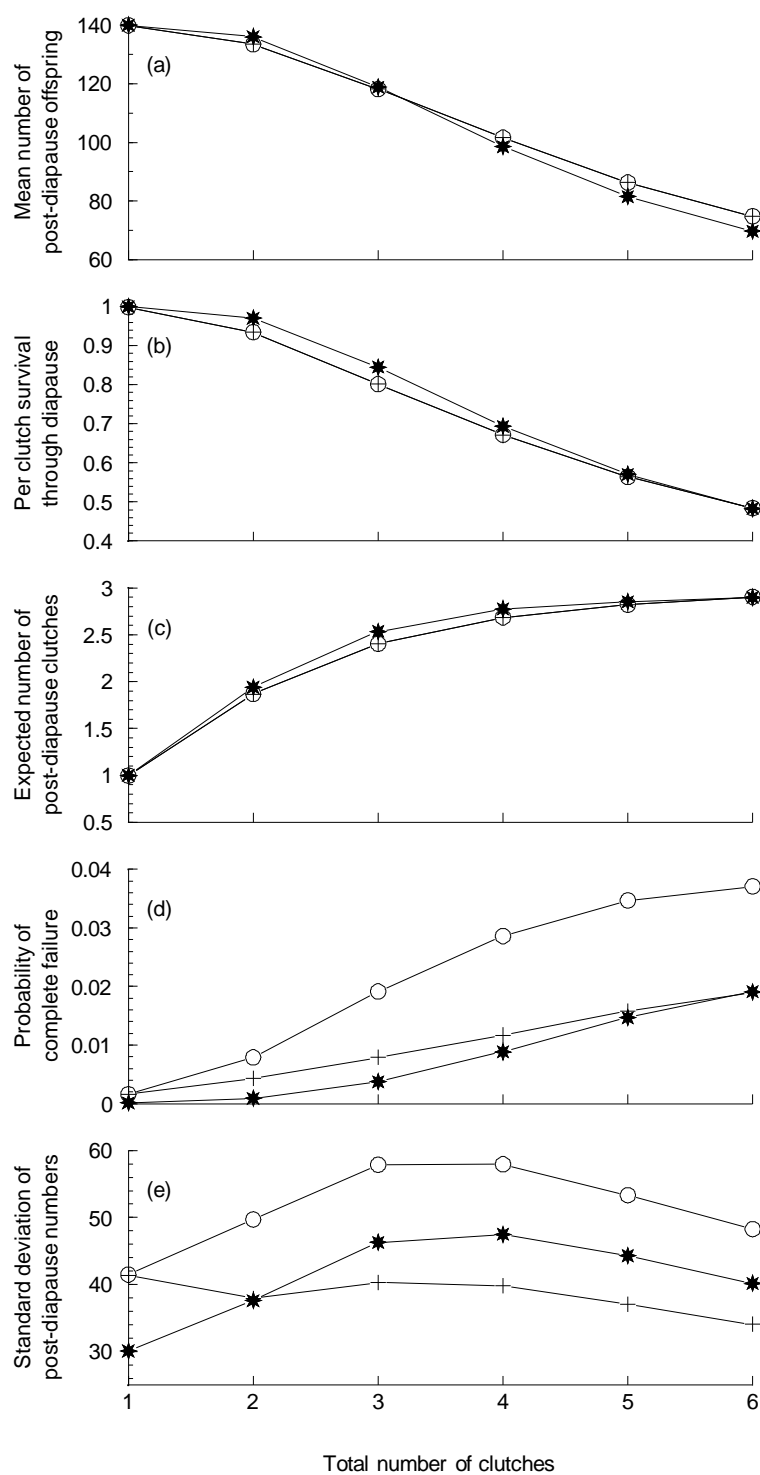


Fig. 3

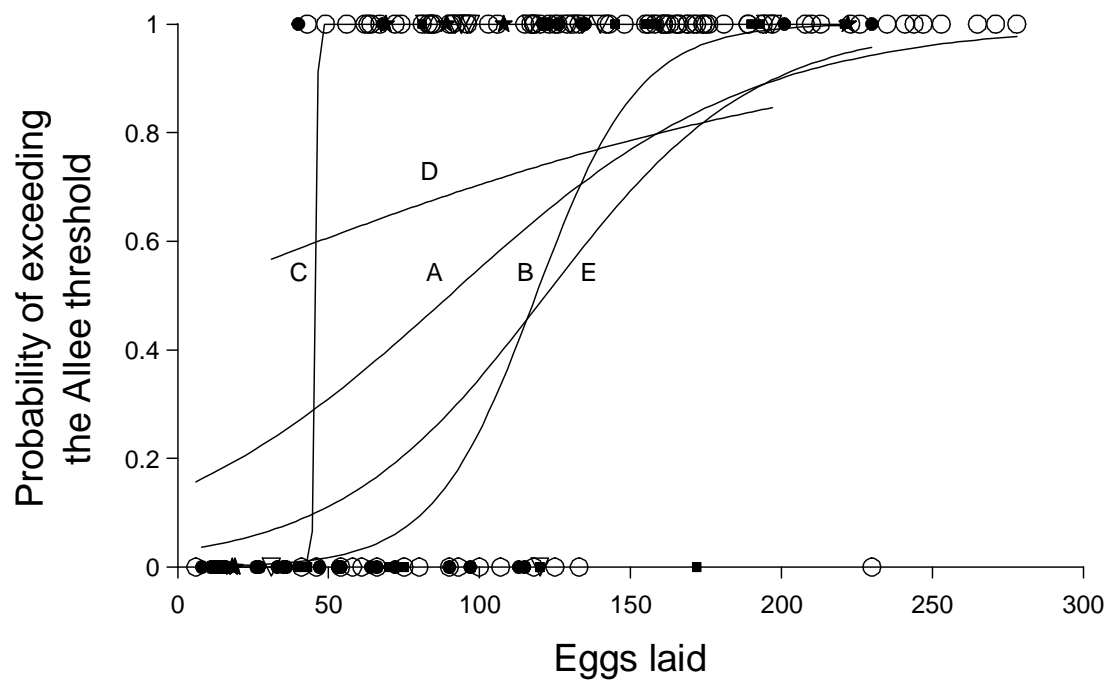


Fig 4

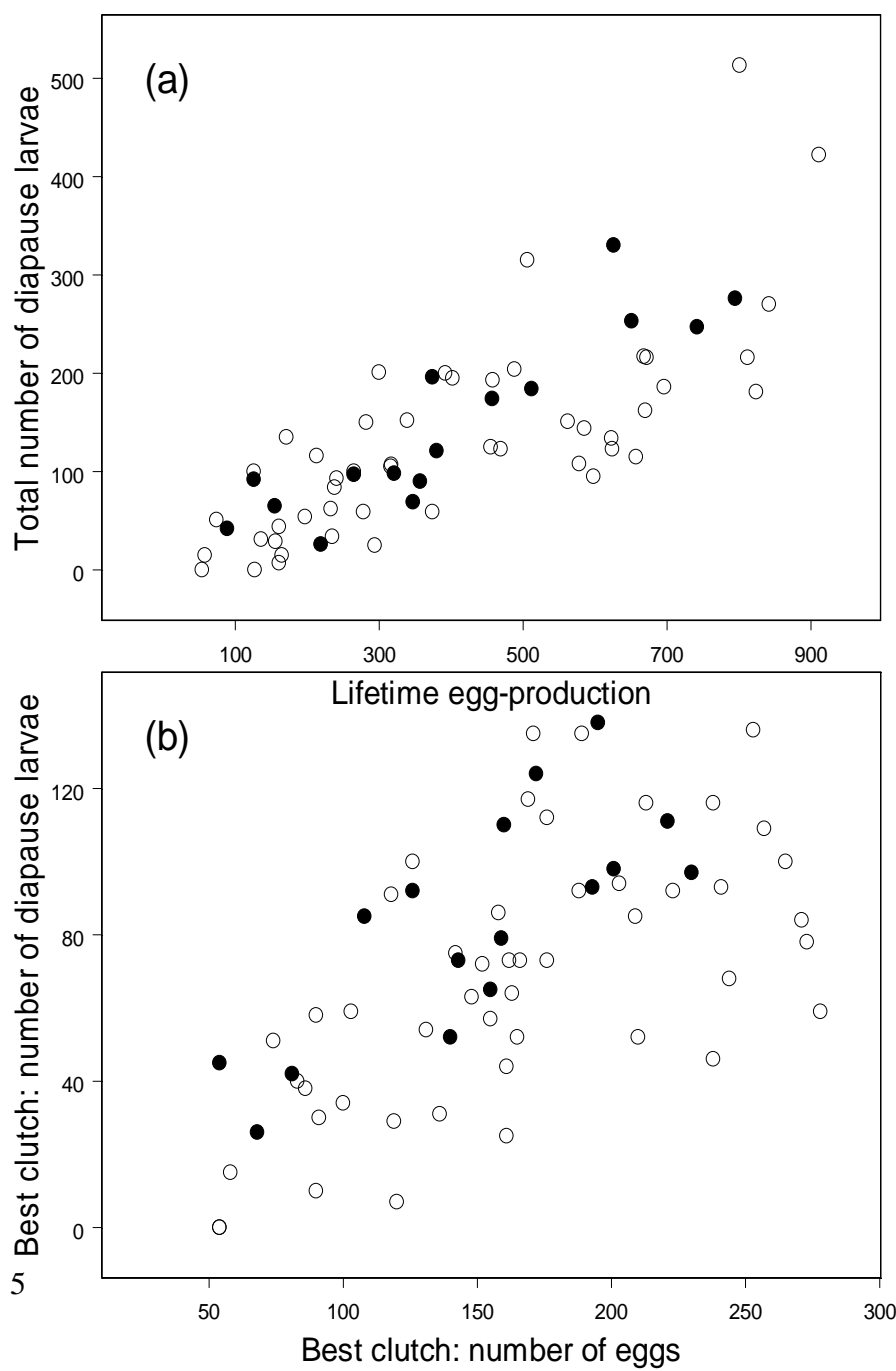


Fig. 5